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A Complex Attenuator Regulates Inducible Resistance to Macrolides, Lincosamides, and Streptogramin Type B Antibiotics in *Streptococcus sanguis*

SUEHARU HORINOUCHI,† WOO-HYEON BYEON, AND BERNARD WEISBLUM*

Department of Pharmacology, University of Wisconsin Medical School, Madison, Wisconsin 53706

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Macrolide-lincosamide-streptogramin B resistance specified by *Streptococcus sanguis* plasmid pAM77 involves an adenine methylase, whose synthesis, demonstrable both phenotypically and by analysis of methionine-labeled proteins made in *Bacillus subtilis* minicells, is inducible by erythromycin, lincomycin, and streptogramin type B antibiotics. Localization of the methylase structural gene, including its control region in DNA fragments obtained with restriction endonucleases, has been deduced from DNA blot experiments with characterized target and probe DNAs from other streptococci, combined with DNA sequence analysis and comparison of the putative streptococcal methylase sequence with that of a cognate methylase in staphylococcal plasmid pE194. The streptococcal methylase migrates electrophoretically in polyacrylamide gels with the mobility of a 29,000-dalton protein. The sequence organization of the putative streptococcal methylase mRNA leader sequence partially resembles its staphylococcal counterpart and can support a similar mechanism of secondary structure rearrangement leading to methylase synthesis. The deduced 5′ leader sequence preceding the pAM77 methylase structural gene sequence comprises approximately 155 nucleotides within which one can identify a putative control peptide 36 amino acid residues in length (in contrast to 19 in the pE194 peptide) and at least 14 possible classes of overlapping inverted complementary repeat sequences (in contrast to 3 in the pE194 control region), one of which can sequester the sequence AGGAG 7 nucleotides upstream from the putative (methionine) start codon of the streptococcal methylase. Comparison of the pAM77 and pE194 methylase amino acid sequences and their respective nucleotide sequences shows 51% conservation of amino acid residues (124 of 244) and 59% conservation of nucleotide residues (433 of 738), which suggests a common origin for the two methylase structural gene sequences. Differences in mRNA base composition associated with conserved amino acid residues occur mostly in the third nucleotide ("wobble") position of codons and may reflect adaptation of methylase genes to optimal expression in host cells with differing codon use patterns.

Plasmid pAM77, isolated from *Streptococcus sanguis* by Yagi et al. (35), specifies resistance to macrolide, lincosamide, and streptogramin type B (MLS) antibiotics. MLS resistance previously studied in *Staphylococcus aureus* systems is mediated by specific N^6-methylation of adenine in 23S rRNA as a result of which ribosomes bind MLS antibiotics with reduced affinity (15, 16, 24, 34). The biochemical details of this mechanism, originally worked out in *Staphylococcus aureus* model systems, were extended to MLS-resistant *Streptococcus faecalis* (8), a species whose MLS determinant cross-hybridizes with that of pAM77 (33).

† Present address: Department of Agricultural Chemistry, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan.

Studies of MLS resistance in *Streptococcus pyogenes* have shown that the specificity of induction in this organism differed from that reported for *Staphylococcus aureus* (4, 14, 17), and preliminary studies in our laboratory indicated that the specificity of induction in *S. sanguis* resembled that reported for *S. pyogenes*. In view of similarity between *S. pyogenes* and *S. sanguis* inducible MLS determinants (both with respect to DNA sequence homology and induction specificity), we have undertaken this study of pAM77 as a model streptococcal system. Introduction of pE194 and pAM77 DNA into *Bacillus subtilis* by transformation makes it possible to study the peptide products synthesized in minicells under plasmid direction and to obtain conveniently ample

TABLE 1. Bacterial strains and plasmids

Strain designation	Relevant plasmid	Relevant phenotype	Derivation	Reference
B. subtilis BGSC 1A197	None	MLS sensitive minicell producer	*spo* mutant, from CU403	Bacillus Genetic Stock Center
B. subtilis(pE194 cop-6)	pE194	MLS resistance	From BGSC 1A197 by transformation	This work
B. subtilis(pAM77)	pAM77	MLS resistance	From BGSC 1A197 by transformation	This work
S. sanguis A1	pAM77	MLS resistance	Natural isolate	2, 35
S. faecalis D5	pAMβ1	MLS resistance	Natural isolate	2

quantities of plasmid DNA for structural studies. Plasmid pAM77 has a relatively low molecular weight, 4.5×10^6 (2, 35), and after introduction into *B. subtilis* it replicates without detectable rearrangement (33), where it confers inducible MLS resistance on *B. subtilis* with the same specificity as in the original *S. sanguis* host. As part of an investigation into the determinants of induction specificity by MLS antibiotics, we report the DNA sequence of the pAM77 MLS methylase, including its putative control region.

MATERIALS AND METHODS

Plasmid DNA preparation. Plasmid DNA from *B. subtilis* transformants carrying pAM77 and pE194 was prepared by cell lysis, using the lysozyme-EDTA method followed by isolation of the covalently closed circular fraction as previously described (13). Covalently closed circular DNA preparations from pAM77 and pE194 were used to transform *B. subtilis*, as described by Dubnau and Davidoff-Abelson (5).

Minicell studies. Minicells of *B. subtilis* strain BGSC 1A197 (a sporulation-deficient mutant of CU403) transformed with pAM77, or with pE194 cop-6, were prepared by centrifugation in sucrose density gradients as described by Mertens and Reeve (22). For analysis of the products of plasmid-directed protein synthesis, minicells were incubated for 30 min at 37°C in 0.5 ml of minimal medium described by Mertens and Reeve (22), supplemented with 50 μCi of [^{35}S]methionine from which unlabeled methionine was omitted, as described by Shivakumar et al. (26). For induction, the incubation medium was supplemented with 0.1 μg of erythromycin, clindamycin, or ostreogrycin B per ml, as indicated. After 30 min of incubation, labeled minicells were collected by centrifugation at 10,000 × *g* for 10 min, washed with minimal medium, and resuspended in 0.1 ml of 0.05 M Tris-hydrochloride (pH 8.0–0.01 M Na_2EDTA–25% (wt/wt) sucrose. A 100-μg portion of lysozyme was added, and the mixture was incubated at 37°C for 15 min, after which the treated minicells were lysed by addition of cracking buffer and resultant proteins were fractionated by polyacrylamide gel electrophoresis, as described before (26).

Enzymes. Restriction endonucleases were purchased from New England Biolabs (Beverly, Mass.) and were used as recommended by the vendor.

Antibiotics. Clindamycin was obtained as a gift from The Upjohn Company, Kalamazoo, Mich. Ostreogrycin B, a streptogramin type B antibiotic, was obtained as a gift from Glaxo Research Ltd., Sefton Park, Stoke Poges, Buckinghamshire, U.K. Erythromycin was purchased from Sigma Chemical Co., St. Louis, Mo.

DNA sequence studies. Nucleotide sequences were determined by the end-label method of Maxam and Gilbert (20). Except for the *Rsa*I site beginning at nucleotide 366, all restriction sites used for end-labeling were verified by determination as part of an overlapping sequence.

RESULTS

Bacterial strains. Relevant strains are described in Table 1.

Induction of MLS resistance in *S. sanguis* carrying pAM77. The specificity of induction of MLS resistance by various MLS antibiotics was tested as described by Weaver and Pattee (31), using cells induced by preincubation with erythromycin, clindamycin, or streptogramin type B antibiotics. All three antibiotics induced resistance to erythromycin, clindamycin, or streptogramin B (Fig. 1). On the other hand, if inducible *Staphylococcus aureus* is similarly induced and challenged, selection of constitutively resistant mutants occurs in the clindamycin and streptogramin B challenge samples after an apparent growth lag of approximately 6 to 8 h (31, 32).

Products made under direction of pAM77 in *B. subtilis* minicells. Minicells carrying pAM77 were induced with MLS antibiotics and incubated with [^{35}S]methionine. Proteins synthesized under these conditions were analyzed by polyacrylamide gel electrophoresis (Fig. 2). For comparison, the products synthesized in *B. subtilis* minicells under the direction of pE194 (cop-6) are also shown. Minicells carrying pE194 were incubated with erythromycin, clindamycin, or ostreogrycin B. Results shown in Fig. 2 (lanes a, b, c, and d) indicate that erythromycin functioned more efficiently as inducer than either clindamycin or streptogramin B. This finding is consistent with studies of pE194 induction

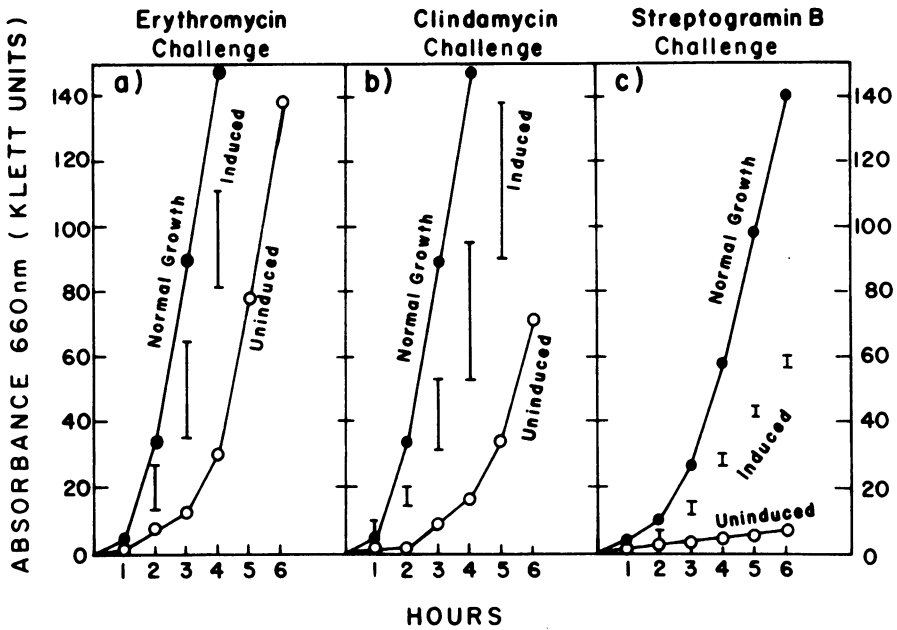

FIG. 1. Induction of *S. sanguis* (pAM77) measured by growth. (a) Induction with 0.1 μg of erythromycin, clindamycin, or ostreogrycin B per ml, followed by challenge with 100 μg of erythromycin per ml. (b) Induction as in (a) followed by challenge with 50 μg of clindamycin per ml. (c) Induction as in (a) followed by challenge with 25 μg of ostreogrycin B per ml. Each panel describes the course of growth in five different cultures, as follows: (i) curves labeled "Normal Growth" describe the course of growth in a culture which was uninduced and unchallenged; (ii) curves labeled "Uninduced" describe the course of growth in cultures which were uninduced, but challenged as indicated at the top of the panel; and (iii), (iv), (v) three curves for which the data points were combined and reported as single vertical bars covering the range of values observed for cultures induced with one of the three MLS antibiotics followed by challenge with the antibiotic named at the top of the panel. Conditions for testing induction were as follows. An overnight culture of *S. sanguis* A1 carrying pAM77 was grown in Todd-Hewitt medium containing erythromycin, clindamycin, or ostreogrycin B at a final concentration of 0.1 μg/ml. The induced overnight culture was used as inoculum and diluted into challenge medium containing the inhibitory concentrations of 100 μg of erythromycin, 50 μg of clindamycin, or 25 μg of ostreogrycin B per ml, as indicated. As controls, uninduced cultures were grown in the presence and absence of challenging concentrations of antibiotics. Results were plotted as absorbance (at 660 nm) as a function of time.

specificity by the antibiotic disk method, in which it was shown that clindamycin and streptogramin B disks produced significant inhibition whereas erythromycin produced a turbid inhibition zone indicative of induction in situ (32, 34). The labeled protein fraction made under direction of pAM77 (Fig. 2, lanes e, f, g, and h) contained five major polypeptide bands, labeled A through E, with mobilities which correspond to molecular masses of 56,000, 36,000, 31,000, 29,000, and 12,000 daltons, respectively. Of these, only the 29,000-dalton component appeared to be synthesized at an increased rate in cells induced with erythromycin, lincomycin, or streptogramin type B antibiotics. The observed molecular weight of the induced band compares favorably with the value of 29,400 daltons predicted by determination of the DNA sequence described below.

Nucleotide sequence of the pAM77 methylase structural gene and its putative regulatory region. Plasmid pAM77 DNA, digested with *Hin*fI restriction endonuclease end-labeled with [γ-^{32}P]ATP and phage T4 polynucleotide kinase, followed by fractionation of the resultant digest by electrophoresis on polyacrylamide gel, yielded seven fragments designated A to G (data not shown). Probes consisting of eluted fragments A to G were tested by the DNA blot hybridization method according to Southern (29), as modified by Smith and Summers (28), using total DNA from *S. faecalis* carrying pAMβ1 digested with *Hin*dIII restriction endonuclease as a target. Of the seven bands tested, only E and F hybridized with the *S. faecalis* target DNA (data not shown). Band E actually was found to contain two fragments unresolved owing to their similar size. To obtain material suitable for sequence analysis, the combined fractions E+E′ were eluted together and digested with *Rsa*I restriction endonuclease, yielding four fragments, completely resolved by polyacrylamide gel elec-

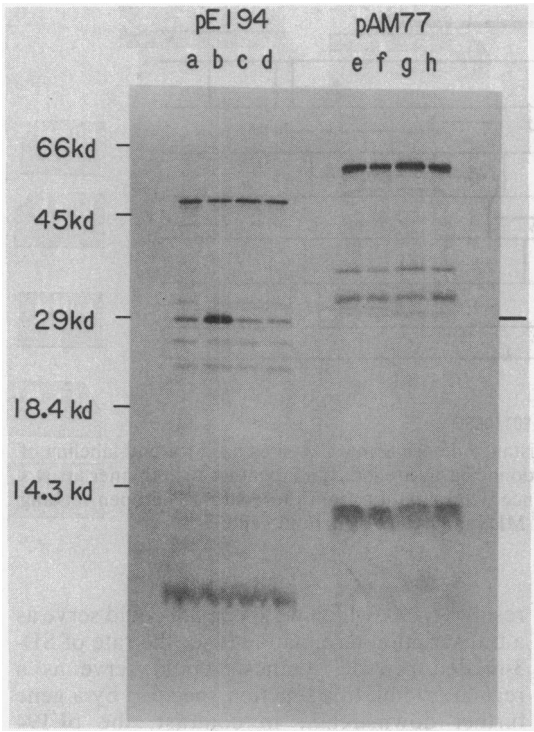

FIG. 2. Products synthesized by *B. subtilis* minicells. (Left) Induction of pE194 methylase: (a) pE194 cop-6, uninduced; induced with 0.1 μg of (b) erythromycin, (c) clindamycin, or (d) ostreogrycin B per ml. (Right) Induction of pAM77 methylase: (e) pAM77, uninduced, (f) pAM77, induced with 0.1 μg of erythromycin, (g) clindamycin, or (h) ostreogrycin B per ml. *B. subtilis* minicells carrying pAM77 or pE194 were incubated with [^{35}S]methionine to label the proteins synthesized and with 0.1 μg of inducer per ml in minimal medium as described previously (26). Lysates were prepared and fractionated by polyacrylamide gel electrophoresis on 15% gels. For both pAM77 and pE194 a single band, 29,000 daltons, appears relatively more intense in samples prepared from induced cultures. If the labeled pAM77 and pE194 samples were mixed and run in the same lane, no separation of the two components in the 29,000-dalton band was detected.

trophoresis, of which bands 2 and 3 (in order of decreasing size) showed positive hybridization and contained the sequence of the pAM77 MLS resistance determinant. Hybridizations were performed in a solution containing 0.45 M NaCl and 0.045 M sodium citrate (3× SSC), also using the modification of Denhardt (3) for DNA-DNA hybridization. We therefore began our sequence analysis with these fragments.

Based on the plan outlined in Fig. 3, the complete DNA sequence of the pAM77 methylase including its putative promoter and control region (Fig. 4) was obtained. Analysis of the DNA sequence shows the presence of an open reading frame between residues 318 and 1,052 capable of encoding a protein containing 245 amino acids with a calculated molecular weight of 29,400. The putative promoter and control regions for the pAM77 methylase resemble those of pE194 in general outline, but with notable differences.

First, the similarities with pE194 include a sequence in the methylase mRNA capable of encoding a control peptide and beginning at nucleotide 115 (Fig. 4) preceded by its Shine and Dalgarno translational initiation sequence GGAGG, labeled SD-1 (25). The methylase structural gene sequence begins at residue 318 preceded by its Shine and Dalgarno sequence AGGAG, labeled SD-2. SD-2 falls within the loop region of the inverted complementary repeat sequence centering on residue 306 (Fig. 4). The extent of this inverted complementary repeat sequence, labeled 14, is shown in Fig. 5, together with its relation to other inverted complementary repeat sequences in the control region. We postulate that induction unmasks SD-2 by ultimately disrupting or preventing the formation of the secondary structure of inverted complementary repeat sequence 14 and permitting the synthesis of methylase. Both pAM77 and pE194 control peptide structural gene sequences use GGAGG (as SD-1), the "strong" Shine and Dalgarno sequence proposed by McLaughlin et al. (21) as that preferentially used by gram-positive bacteria for initiation of protein synthesis. In contrast, both pAM77 and pE194 use, as SD-2, sequences which would be expected to pair more weakly with 16S rRNA than SD-1.

The control region of pAM77 differs from that of pE194 with respect to the length of the control peptide and the inverted complementary repeat sequences whose secondary structure determine the rate of methylase synthesis. The pAM77 control peptide sequence codes for 36 amino acids, whereas the corresponding pE194 peptide sequence codes for 19. We can identify at least 13 successive centers of symmetry of inverted complementary repeat sequences in the pAM77 control region (Fig. 5). These inverted complementary repeat sequences, collectively comprising 228 nucleotides between residues 120 and 348, cover the coding region for the last 33 amino acids of the control peptide and the coding sequence for the first 9 amino acids of the methylase. The 14 inverted complementary repeat sequences would enable the pAM77 control region to assume a correspondingly larger number of alternative paired conformational states than the pE194 control region. The corresponding pE194 region, in contrast, contains only three clearly defined centers of symmetry, which in turn could support five different conformational states falling into two distinct classes,

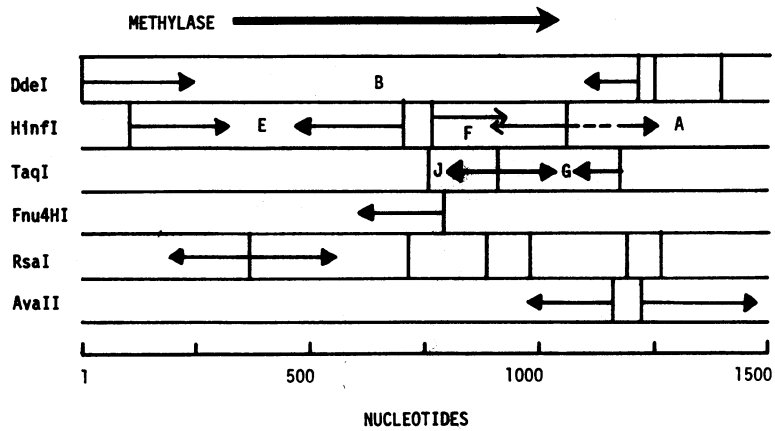

FIG. 3. Sequencing strategy for the pAM77 MLS resistance determinant. The sites used for end-labeling of DNA fragments obtained by digestion with restriction endonucleases are indicated together with thinner arrows whose length and direction indicate the extent of sequence determination from these sites. The open reading frame (thicker arrow) indicates the extent of the pAM77 MLS methylase structural gene.

active and inactive, as shown previously (9, 11, 12). In addition, the inverted complementary repeat sequences in pE194 are 12 to 14 nucleotides in length, each perfectly paired except for a single nucleotide bulge.

The pAM77 inverted complementary repeat sequences all appear highly interrupted. This feature, together with the twofold greater number of codons at which ribosomes can potentially stall in the pAM77 control region, may account for the greater number of MLS antibiotics capable of inducing this system. According to the model which we propose for the pAM77 attenuator, the inducing antibiotic can cause ribosomes to stall preferentially at specific locations in the control peptide coding sequence (residues 115 to 222) during translation of the pAM77 methylase mRNA. This stall effectively traps inverted complementary repeat sequences 1 to 6 in their entirety, as well as the 5′ arms of inverted complementary repeat sequences 7, 8, and 9 and possibly parts of the 5′ arms of 10 and 11. To the extent that inverted complementary repeat sequences 11, 12, and 13 can form associated hairpin structures, inverted complementary repeat sequence 14 is prevented from forming, leaving SD-2 accessible for formation of initiation complexes with ribosomes.

The two methylase structural genes show significant differences at their 3′ termini. The pAM77 methylase structural gene terminates at residue 1,055 (Fig. 5). At residue 1,045 a potential Shine and Dalgarno sequence GGAGG (SD-3) precedes the sequence of 129 nucleotides beginning at 1,055 capable of encoding a 43-amino acid peptide. The hypothetical C-terminal peptide coding sequence terminates in an inverted complementary repeat sequence centering on residue 1,190, which we speculate could serve as a transcription terminator. If so, the rate of SD-3-related peptide synthesis could serve as a regulatory link to a function specified by a gene further downstream. In contrast, the pE194 methylase structural gene appears to terminate at the beginning of an inverted complementary repeat sequence capable of serving as transcription terminator by analogy to similar sequences found in other systems (23, 27). Additional inverted complementary repeat sequences centering on residues 1,290, 1,342 to 1,343, and 1,385 to 1,386 are present (see Fig. 4). The functional significance of these symmetric sequences is not apparent.

Comparison of the pAM77 and pE194 methylase sequences. The two methylase structural genes can be compared with reference to their respective nucleotide and peptide sequences (Fig. 6). The pAM77 methylase contains 245 predicted residues, whereas the pE194 methylase contains 244. Of these, 122 amino acid residues are identical in sequence. Likewise, 433 of 738 residues (59%) of the nucleotides are sequentially identical. In the case of amino acid residues 163 to 172, an unbroken stretch of nine amino acids is conserved in both methylase sequences. Additional clusters of identical amino acids can be seen as well as amino acid sequences for which chemical properties of the side chain are conserved. The structural similarities of the streptococcal and staphylococcal methylases suggest that they are related to a common ancestral gene.

Codon use in the streptococcal and staphylococcal methylases. The base compositions of *S. sanguis* and *Staphylococcus aureus* DNA have been determined and reported to fall in the 38 to

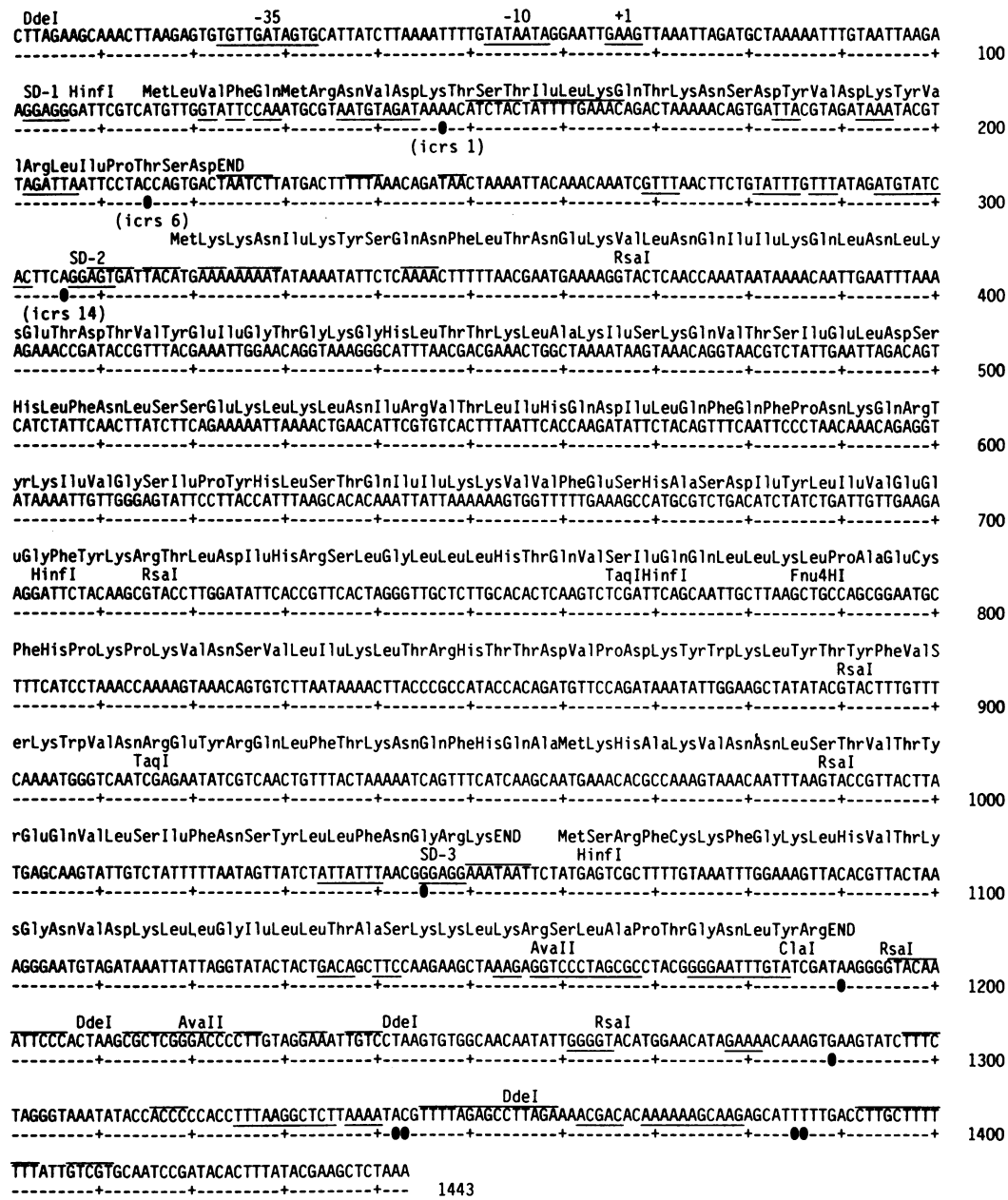

FIG. 4. The nucleotide sequence of the pAM77 MLS resistance determinant from a *Dde*I site, as indicated in Fig. 3, is shown together with the potential amino acid sequences specified by open reading frames deduced from the DNA sequence. Inverted complementary repeat sequences are underlined and their centers of symmetry are indicated with solid circles. Inverted complementary repeat sequences 1, 6, and 14 are shown spanning the control region. Additional inverted complementary repeat sequences associated with the control region are shown in Fig. 5. The inverted complementary repeat sequence comprising residues 1,157 to 1,223 overlap the C-terminal portion of a hypothetical peptide whose coding sequence, beginning five nucleotides after the methylase translation terminator codon, may form part of a functional tandem attenuator, as discussed in the text.

40 mol% guanine-plus-cytosine (G+C) range for the former and 32 to 34 mol% G+C for the latter (19). Codon usage in the two methylases would be expected to reflect adaptation to this difference in DNA base composition. Comparison of the base composition of the streptococcal and staphylococcal methylase structural gene sequences shows that the average percent G+C for codons used is 36 and 29% G+C, respectively (Fig. 6). Examination of codons for the 124-

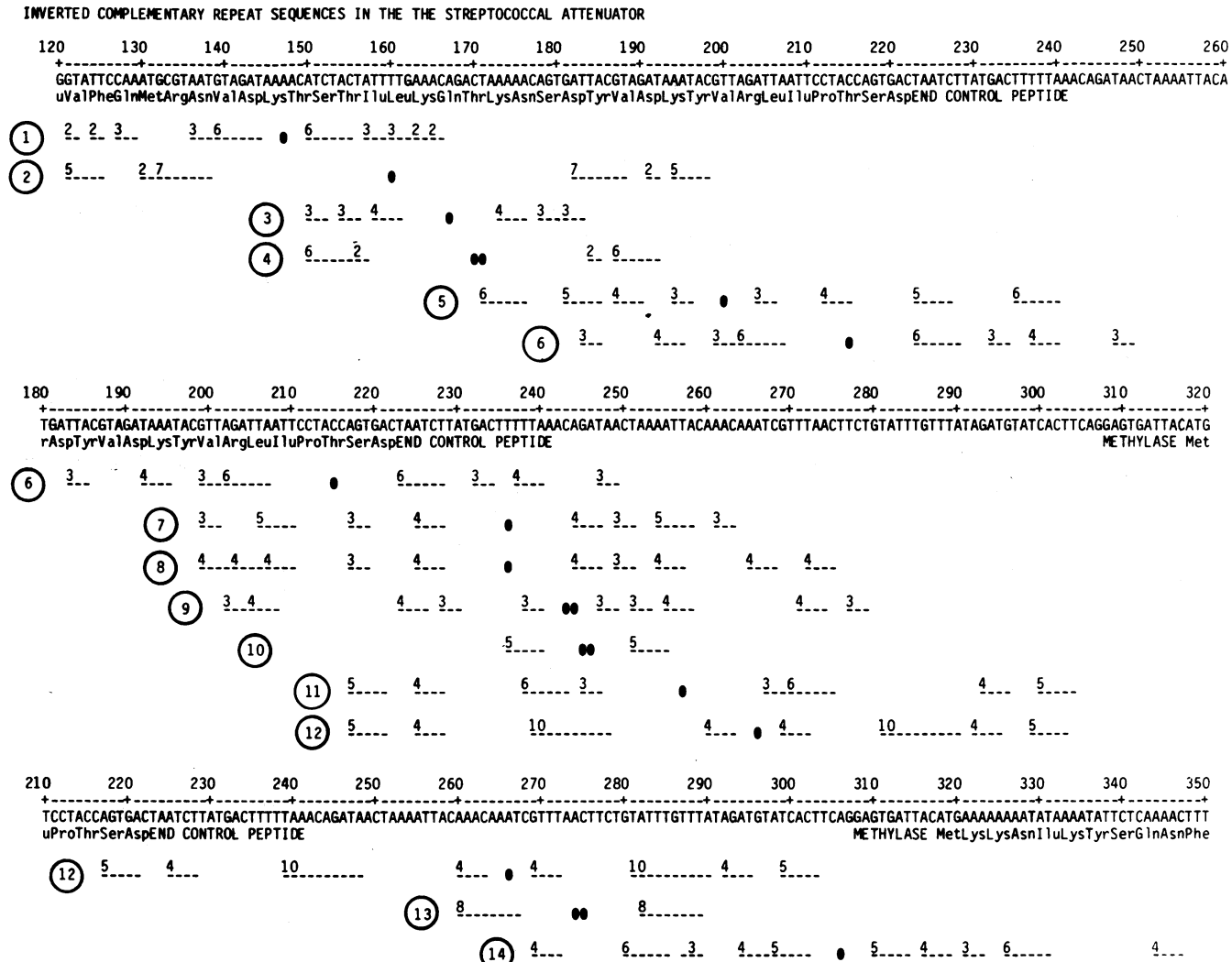

FIG. 5. Potential patterns of inverted complementary repeat sequence associations in the pAM77 control region are numbered 1 through 14. Disruption of the secondary structure by ribosome stall over the peptide coding sequence (residues 115 to 222) completely blocks inverted complementary repeat sequences 1 to 6. By partially blocking the 5′ arms of inverted complementary repeat sequences 7, 8, and 9, the 3′ arms of inverted complementary repeat sequences 11 to 13 pair preemptively with residues through 302, preventing formation of inverted complementary repeat sequence 14 and resulting in unmasking of the Shine and Dalgarno sequence at position 308.

COMPARISON OF THE NUCLEOTIDE AND AMINO ACID SEQUENCES OF 2 MLS DETERMINANTS

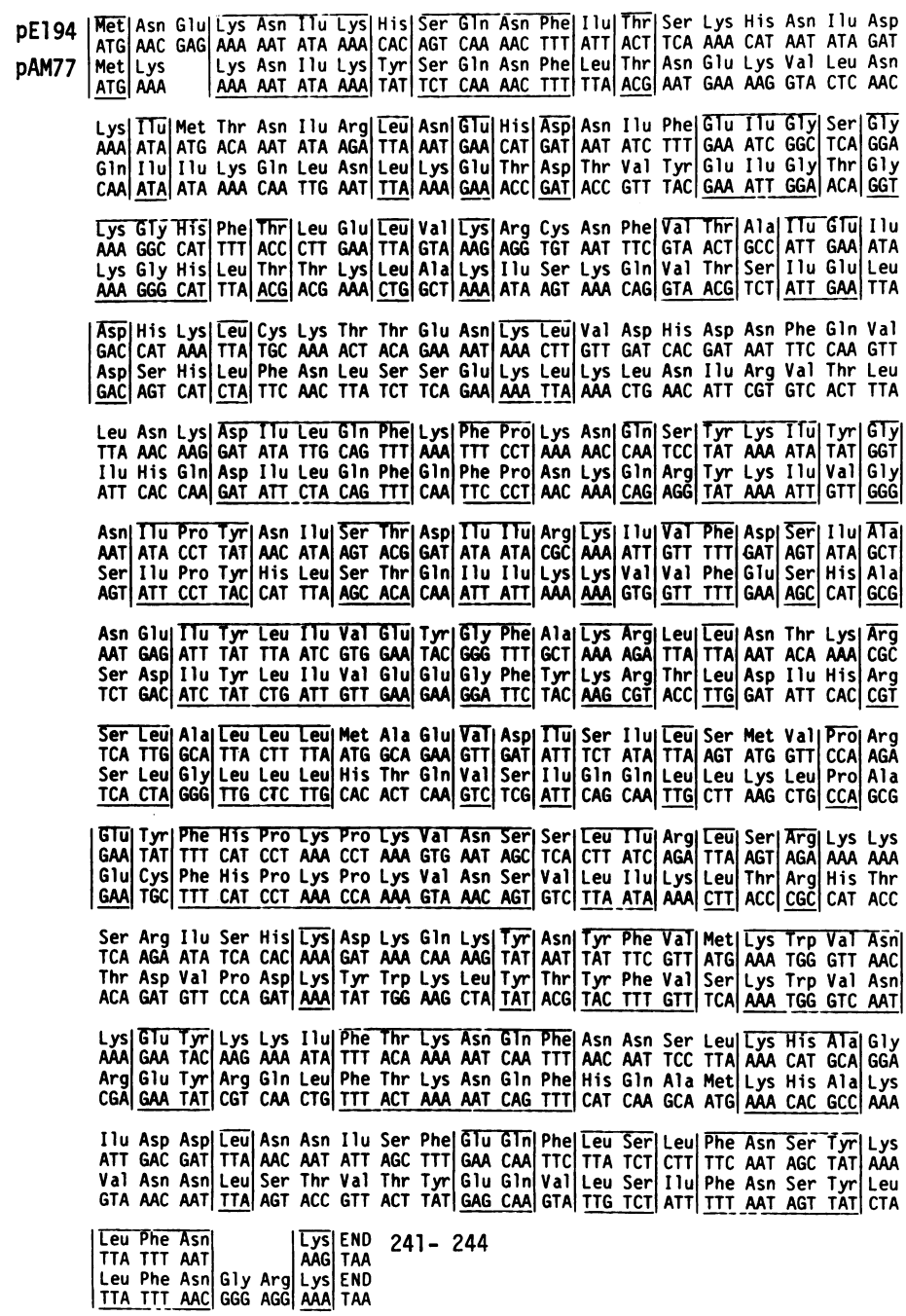

FIG. 6. Comparison of the pAM77 and pE194 methylases and codons. The methylase coding sequences of plasmids pAM77 and pE194 are displayed in parallel, indicating regions of identity in the polypeptide sequence. In many instances it is noted that the amino acid has been conserved, although codon usage differs.

amino acid residues identical in both proteins shows that 50 are specified by identical codons. Of the remaining 74 identical amino acids with different codons, 51, the majority differ in the third nucleotide ("wobble") position. For the 51 codons differing in the third position, 25 involve a change of either adenine or thymine to G or C, 17 involve a change of either G or C to either adenine or thymine, and 9 involve no net change in base composition in going from pE194 (lower percent G+C) to pAM77 (higher percent G+C). These findings are consistent with the interpretation that the streptococcal and staphylococcal methylases are related and that they have diverged from a common ancestral sequence.

DISCUSSION

MLS resistance occurs in a wide variety of clinical isolates of streptococci (2). Previous studies from our laboratory have shown that the streptococcal resistance determinants show homology to each other and to the MLS determinant in *Staphylococcus aureus* plasmid pI258 (32). Plasmid pAM77 provides an opportunity to examine interspecies distribution of apparently related antibiotic resistance determinants and the extent to which models of gene regulation by translational control, similar to the model postulated for the regulation of MLS resistance in pE194 (9, 11, 12), can be found in other organisms. Moreover, we are challenged to explain (i) the lack of sequence homology found in hybridization experiments comparing the MLS determinants of pAM77 and staphylococcal plasmid pE194, and (ii) differences of induction specificity wherein clindamycin and streptogramin type B antibiotics both induce the pAM77 determinant but neither induces the pE194 determinant, in contrast to erythromycin which induces both determinants. Ultimately, it would be desirable to extend these findings to explain the wide diversity of induction phenotypes found in *Streptomyces* spp. (7), in which a subset of MLS antibiotics (e.g., tylosin and ostreogrycin B) incapable of inducing *Staphylococcus aureus* appear capable of inducing some, but not all, inducible *Streptomyces* spp.

Comparison of the DNA and respective amino acid sequences of pAM77, as a representative of the pI258-streptococcal family, with pE194 strongly suggests that these sequences are related, that at least part of the relatedness can be localized to regions which code for methylase, and that observed lack of homology by the hybridization method can most likely be ascribed to probe-target hybrid instability resulting from 41% sequence mismatch which we have shown experimentally in this work.

In our initial studies to locate the pAM77 MLS resistance determinant, we noted that probes prepared from pAM77 *Hin*fI fragments E and F showed cross-homology with plasmid pAMβ1 DNA from *S. faecalis*. Since fragments E and F contain methylase coding sequences, we infer that at least part of the sequence homology observed between pAM77 and the other streptococcal DNA blots shown previously is due to structural gene sequence homology. Our findings leave open the question of the extent of transposon and control region sequence homology. At this point, the control regions for the pAM77 and pE194 methylase determinants appear to show less similarity than the structural gene coding sequences.

The MLS control regions from pE194 and pAM77 show markedly different patterns of inducibility. Only a limited number of MLS antibiotics have inducing activity in pE194, whereas in pAM77 all MLS antibiotics appear to have inducing activity. We postulate that this difference may be related to the larger control peptide found in the pAM77 methylase insofar as it presents twice as many codons as potential sites for ribosome stall in the presence of inducing concentrations of MLS antibiotics. Moreover, the large number of alternative partially destabilized conformations which the pAM77 control region can assume would tend to diminish the significance with which any single conformation, destabilized by mutation, can effectively result in constitutive expression. Consistent with this is the experimental finding that we have not been able to select spontaneous constitutively resistant mutants of pAM77 by the usual techniques successfully used for selection in *Staphylococcus aureus*, namely, selection using MLS antibiotics which do not induce in the particular system. This temporarily precludes critical tests of the pAM77 attenuator model by mutational analysis similar to that described previously for the pE194 attenuator (11, 12).

In our previous studies (32) it was shown that pE194 introduced into *B. subtilis* conferred inducible MLS resistance as in the original host, *Staphylococcus aureus*. From this observation and subsequent subcloning experiments (11, 13), we inferred that the information needed for inducible MLS resistance was contained in pE194 *Taq*I fragment A and that host cell contributions to expression of MLS resistance, if any, were general rather than specific.

In the present study, we show that two different MLS plasmids from different backgrounds with different induction specificities, introduced into *B. subtilis*, confer an induction phenotype which resembles that of the host strain in which the plasmid originally resided. From this observation we infer that induction specificity is a function encoded by the plasmid DNA and that it is determined by the amino acid sequence of

the control peptide. The control peptide is otherwise unnecessary for further expression of MLS resistance since a deletion mutant was obtained in which the coding sequence for this peptide was deleted with retention of the MLS resistance phenotype (12).

Malke and Holm (18) and Hardy and Haefeli (10) have reported that MLS resistance can be expressed in *Escherichia coli*, and in a study of induction specificity Hardy and Haefeli (10) noted that in *E. coli* clindamycin appears to induce more effectively than erythromycin, as determined by the disk assay. Thus, the same determinant in two different host backgrounds can also express two different phenotypes. We feel that this latter observation does not negate our hypothesis but points to systematic differences between ribosomes from gram-positive and gram-negative organisms. Some of these have been summarized by McLaughlin et al. (21) with reference to the specificity of the initiation step in protein synthesis.

Comparison of the sequence organization of the pAM77 and pE194 determinants revealed an unexpected feature not found in the pE194 determinant. The inverted complementary repeat sequence region between residues 1,134 and 1,240 in the sequence shown in Fig. 4 with its associated peptide coding sequence is compatible with possible transcriptional attenuator function. This hypothetical 43-amino acid peptide sequence appears to serve no obvious function that might regulate methylase synthesis in pAM77, and examination of the pattern of induced minicell products synthesized by *B. subtilis* under direction of pAM77 in Fig. 2 reveals increased synthesis of only the 29,000-dalton component in the fractionated mixture of minicell proteins. We speculate that the hypothetical peptide encoded by nucleotides 1,060 through 1,188 could represent a fragment of a second attenuator in tandem with the methylase attenuator.

Tomich et al. (30) have reported that erythromycin induced both expression of MLS resistance and transposition of Tn*917* carrying the MLS determinant from *S. faecalis* plasmid pAD2 to a coresident hemolysin plasmid, pAD1. A putative transposase messenger sequence downstream from the methylase could be induced by erythromycin if it had its own transcriptional or translational attenuator. More generally, the presence of additional attenuators, either transcriptional or translational, could serve to activate entire sets of genes and to coordinate their expression in time. Coordinately regulated genes under translational attenuation control distributed throughout the genome need not be part of the same transcriptional unit. Such a model could serve for temporal control of a wide range of genes whose expression is needed at a defined stage in the life cycle of the organism, for example, antibiotic production or sporulation in *Streptomyces* spp. Ribosome stall in response to endogenous or exogenous factors which inhibit protein synthesis could serve as the common feature of such temporal control signals, and variations in control peptide sequences could determine the relative level of gene expression.

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